Annual Progress Report

Limiting Factors in the Mass Culture of Unicellular Algae

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Introduction

The progress report submitted herewith covers Project Nonr 556 (oo), "Limiting Factors in the Mass Culture of Unicellular Algae", for the period January 1, 1953 to December 31, 1953. Co-principal Investigators are: Hugh G. Gauch and W. M. Dugger; assistants are: Robert W. Krauss and William H. Thomas. The annual operating budget is $4,700.00.

Objectives

There are two objectives of the study. First is the determination of the factors which may limit the continuous and efficient utilization of light by algae growing in mass cultures. This involves a study of whether the theoretical photosynthetic efficiency of the algae can be attained and sustained in mass culture apparatus. Second, and complementary to the first objective, is the study of the changes in the metabolism of the algae which may take place as culture conditions are changed. This latter information will establish what compounds can be produced in the algae and is essential if a qualitative as well as a quantitative estimate of growth is to be obtained.

No attempt will be made to review here the secondary objectives and possible implications of this study. It is sufficient to mention that by investigating the factors affecting algal growth it is hoped that the information obtained will be pertinent to the problems concerned in exploitation of their photo- and chemo-synthetic potential as well as to the comprehension of their role in the economy of the rivers, lakes, and oceans.

Abstract of Results

(a) Since the start of the project

In order to understand the approach made to the problem it is helpful to review briefly the techniques employed in this study. It was first desirable to utilize a culture vessel large enough to permit adequate and frequent removal of samples of both the algae and the medium without seriously disrupting the growth cultures. Such a culture must be large enough to render realistic extropalations from experimental to actual growth in natural bodies of water or pilot-plant apparatus. Furthermore, the artificial light sources employed, to give constant illumination to the culture, must be as near to the quality and intensity of sunlight as practical. The apparatus designed to provide these requisites consists of two 300-liter vats enclosed in an air-conditioned chamber and illuminated by combined batteries of fluorescent and incandescent lamps. The alga employed in all experiments has been the auto-troph, *Scenedesmus obliquus*.

A basic assumption in the conduct of the experiments has been that if the physical and chemical environment were maintained constant and at an optimum level the growth rate or yield of algae would remain constant and optimum. Because light is the critical physical limiting factor, early experiments were directed toward determination of the cell density which would permit the maximum yield. This was established to be at a dry cell weight of 0.150 gm per liter of medium. At this density the growth rate ,K, is 1 $\log_2$ unit per day which means a doubling of the cell count or weight every 24 hours. As is pointed out in the paper, The Growth and Inorganic Nutrition of *Scenedesmus obliquus* in Mass Culture by Krauss and Thomas, this rate is much less than

the maximum for Scenedesmus. This apparent anomaly is resolved by considering two factors in the growth of the algae. First, the growth rate which is at an optimum near the start of the culture is gradually reduced as the increasing cell density reduces the amount of light intercepting each cell. Second, though the growth rate may be falling the increasing number of cells which can divide permits the yield per unit time to be optimum at a cell density that is too high to allow for maximum growth rate. This means that if the cell density is maintained at the calculated level for optimum yield, by harvesting the increment, light will be constant per unit cell and the yield should be constant.

With the requirement for maximum light utilization satisfied, attention was turned to the capacity of the medium to sustain growth over a protracted period of time. Because algae cultured in a system subjected to repeated or continuous harvest progressively remove nutrients from the medium it is essential to establish the rate of uptake of each element. This requires inorganic analyses of the harvested cells. The rate of removal can them be the basis for return to the medium of the major elements removed. These analyses were made on cells from a number of mass cultures and constitute the present basis for replenishment in media sustaining continuous harvest.

The experiments and data obtained in these investigations has been reviewed in ONR Progress Reports for the year 1952 and in detail in the paper, Growth and Inorganic Nutrition of Scenedesmus oblequus in Mass Culture forwarded to the publisher, Plant Physiology, and to the Office of Naval Research in March of 1953.

(b) For the current report period

The work summarized in part (a) above provided necessary information for maintaining both the physical and chemical environment at an optimum level for growth. It supplied what appared to be the information necessary to permit continuous mass culture and constant yields. As a result emphasis during the current report period has been directed at two questions. First, does the recycling system which was developed allow for continuous uninterrupted growth or do other factors limit even in view of an apparently adaquate physical and chemical environment? Second, what information can be obtained concerning the changes in the major inorganic element, nitrogen, as the environment is changed?

Initial progress in answering these two questions is reviewed in the ONR Progress Report dated 15 July 1953 as required under the previous report schedule. This information will not be repeated in this report except as it is necessary to make the abstract coherent. For convenience the results of the investigation of the two problems will be reported under two headings- Nutrition, and Nitrogen Fractions.

Nutrition

During the experiments run for the purpose of determining the rate of uptake of the macronutrients- nitrogen, phosphorous, sulfur, potassium, and magnesium- it became apparent that the magnified demand of the culture for micronutrients would necessitate a technique for resupply of these nutrients as well. Therefore, experiments were performed to determine the best methods for supplying iron, manganese, zinc, cobolt, copper, and calcium. Chlorides

and sulfates of these elements added periodically during culture were compared to chelate complexes of the same elements added only initially to the medium. When parallel, continuously harvested cultures were grown, growth in the culture supplied the metals in chelate complex with ethylenediaminetetraacetic acid exceeded by 22% that in cultures without the chelating agent. The difference was not as great during the early period of growth but became more conspicuous as the culture aged.

It is not possible, nevertheless, to evaluate with precision the effectiveness of a system of micronutrient supply unless it is possible to measure the demand for these metals exerted by the cells. This problem was met in two ways. First, samples of the algae grown in cultures supplied micronutrients as salts and as chelate complexes were ashed and submitted to spectrographic analysis. The result of these analyses are as follows:

Table I

Micronutrient content of Scenedesmus grown in media supplied micronutrients chelated by EDTA compared to Scenedesmus grown in media supplied micronutrients as chloride, sulfate, and nitrate salts.

Given as percent dry weight

	-EDTA		+EDTA	
Element	1st Harvest	10th Harvest	1st Harvest	10th Harvest
Fe	0.0572	0.0428	0.0788	0.5624
Ca	0.0827	0.0027	0.0512	0.0045
Mn	0.0093	0.0016	0.0096	0.0078
Zn	0.0014	0.0023	0.0023	0.0045
Cu	0.0014	0.0012	0.0039	0.0021
Co	0.0001	0.0003	0.00004	0.00006

The results of these analyses indicate that the micronutrient formula previously used was inadaquate in supplying both calcium and manganese to sustain growth. They further indicate that though EDTA was apparently satisfactory in providing most of the micronutrients iron was precipitated as the culture aged. This may be the primary cause of reduced growth in older cultures.

This work, however, did not determine what levels of chelated micronutrients would be sustained by the cells. Therefore a series of experiments to determine these levels was performed in a multiple culture apparatus developed under a grant from the Carnegie Institution of Washington. This apparatus permits small cultures to be grown under aseptic conditions and allows for close control of the environment. The results of these experiments are given in Table II.

Table II

Growth of Scenedesmus in flask cultures showing response to different levels of chelated mirconutrients.

Percent of optimum concentrations

Metal ppm	Fe	Ca	Mn	Zn	Cu	Co
0	01.0	72.0	18.7	100.0	100.0	63.5
3	95.0	100.0	87.5	100.0	100.0	100.0
9	100.0	64.0	100.0	73.7	89.5	69.5
27	36.5	38.9	62.5	79.0	79.0	61.5
81	0.0	27.7	6.2	84.2	68.5	55.0

These experiments established the micronutrient levels sustaining optimum growth under conditions of pure culture and indicate the concentrations which can be expected to give best results in mass cultures.

Many intriguing problems concerning toxicity and sufficiency levels of the micronutrients have presented themselves during the course of this investigation. A new chelating agent, dihydroxyethylene-diacetic acid, which prevents precipitation of iron has been tested. A species of Penicillium has been isolated which uses the supposedly non-metabolizeable EDTA as a source of carbon and possibly nitrogen. With this in mind we are screening a series of antibiotics to determine the feasability of preventing deterioration of a non-sterile medium in mass culture. Intensive study of these problems is anticipated during 1954. The next phase, however, will deal with the response of the algae to the new method of micronutrient supply developed during the past fall. It is hoped that not only will we be able to obtain a sustained yield in mass culture but that the daily yield may be increased as well.

Nitrogen Fractions

Not only does the mass culture technique offer a means for studying algal growth but it provides a unique opportunity for a direct analytical study of the metabolic status of the cells produced. The analytical approach is being employed to study the course of nitrogen metabolism in Scenedesmus. Algas grown in a complete nutrient solution are harvested and transferred to media defficient in one or more essential elements. Changes in the nitrogen metabolism can then be followed by periodic analysis. Not only can different media be used but the amount of light reaching the cell can be varied at will. In brief the analytical procedure for the samples is as follows:

Portions of each sample are taken for total nitrogen determination and for dry weight measurements and the remainder of the sample (10-15 grams fresh weight) is extracted with hot 1% acetic acid and water. This precipitates the protein and releases soluable nitrogen from the cells, Chemical analyses are performed on the cells as follows: total N, by standard micro-kjeldahl procedure on the whole cells; total soluble N, by a micro-kjeldahl procedure on an aliquot of the extract; protein-N, the difference between total-N and total soluble-N; free amino-N, by either a manometric or photometric ninhydrin procedure; ammonia-N, by vacuum distillation at pH 8.5, followed by Nesslerization; total amide-N, by hydrolysis in 1N H_2SO_4 at 100° C. for three (3) hours, followed by an ammonia determination; combined amino-N, by hydrolysis in 6N HCl for 24 hours at 110° C., followed by an amino N determination; basic-N, by precipitation with phosphotungstic acid, followed by a total N determination on the precipitate and an amino-N determination on the filtrate; nitrite N, by a photometric procedure using the reaction of nitrite with alpha-naphthylamine and sulfoilic acid; nitrate-N, by a phenoldisulfonic acid photometric procedure; arginine, by a photometric Sakaguchi procedure. Paper chromatographic methods will be described later.

Nitrogen deficiency

The first deficiency studied was nitrogen deficiency. Five days after the cells were transferred to a nitrogen deficient medium, the total nitrogen had decreased from an initial 7.5% to 2% of the dry weight (see Table III).

Changes in the nitrogen fractions of Scenedesmus in a nitrogen deficient medium.

Table III

Fractions	Days in deficient medium									
	0	1/3	2/3	1	2	3	5	8	11	14
	% Dry weight									
Total N	8.50	6.38	6.06	4.68	3.42	2.84	2.15	2.08	2.01	2.07
Protein N	6.95	5.88	5.65	4.36	3.22	2.65	2.03	1.95	1.87	1.95
Soluble N	0.55	0.50	0.41	0.32	0.20	0.19	0.12	0.13	0.14	0.12
Free amino N	0.12	0.10	0.06	0.04	0.03	0.03	0.03	0.03	0.03	0.02
Amide + NH_3 N	0.01	0.03	0.01	0.02	0.01	0.01	0.01	--	--	0.01
Combined amino N (Peptides)	0.05	0.10	0.11	0.09	0.02	0.03	0.03	--	0.03	0.05
Basic N	0.05	0.05	0.06	0.04	0.03	0.02	--	--	--	0.02

No major shifts in the proportion of the various fractions were observed during this decrease. Protein continued to comprise 93% of the total nitrogen. Growth continued at about 50% of its initial rate and presumably nitrogen was being redistributed among the daughter cells, thus accounting for the decrease in total nitrogen expressed as per cent dry weight. This hypothesis proved to be only partially true as was shown in another experiment in which the total amount of nitrogen in the culture was determined at different stages of nitrogen deficiency. The results of this experiment are given in Table IV.

Table IV

Loss of nitrogen from Scenedesmus cells placed in a nitrogen defficient medium.

Days N Deficient	Total N of cells % dry Weight	Total N of cells in gm/300 liters	Loss gm/300 liters
0	7.07	5.80	0.00
0.5	5.87	5.22	-0.58
1	5.67	5.82	+0.02
2	4.00	5.29	-0.51
4	2.53	4.76	-1.04
9	1.69	3.93	-1.87

At the end of nine days spproximately a third of the total nitrogen in the cells had been lost. Either this nitrogen was present in an unavailable form in the culture solution or had been completely lost.

When deficient cultures were resupplied with nitrogen in the form of potassium nitrate, the total cell nitrogen increased very rapidly, rising from 2% to 8% in 24 hours. The nitrogen fraction data are shown in Table V.

Table V

The effect on the nitrogen fractions of resupplying nitrogen as potassium nitrate to nitrogen-deficient <u>Scenedesmus</u> cells.

	% dry weight								
	Hours after NO_3 addition as KNO_3								
	0	0.5	1	2	4	8	12	24	48
Total N	2.06	2.11	2.13	2.25	2.54	3.59	4.98	7.93	8.26
Protein N	1.95	2.00	1.98	2.10	2.20	2.84	3.96	6.88	7.70
(% of Total N)	94.6	94.6	93.1	93.4	86.6	29.2	79.5	86.8	93.2
Soluble N	.116	.106	.152	.174	.346	.751	1.018	1.042	.551
Nitrate N	.007	.003	.009	.007	.012	.028	.038	.039	.036
Basic N	.046	.043	.057	.049	.146	.426	.598	.603	.225
Amino Basic	0	-	0	.017	.044	.040	.095	.099	.070
Non-Amino Basic	.046	.043	.057	.032	.102	.386	.503	.504	.155
Arginine Guanido N	.009	.007	.011	.030	.129	.345	.553	.458	.027
Amide N	.003	.004	.025	.007	.010	.033	.026	.013	.011
Ammonia N	.003	.002	.002	.002	.002	.002	.004	.003	.002
Free Amino N	.019	.026	.032	.048	.070	.085	.105	.132	.088
Combined Amino N	-	.019	-	-	.071	.158	.192	.197	.127

During the first 2 hours, no shift in the proportions of the fractions was observed. Protein nitrogen continued to account for 93% of the total nitrogen. A slight increase in free amino and amide nitrogen was observed fter one hour. After four hours, however, protein synthesis lagged behind the increase in total nitrogen. This lag was not drastic, since the minimum protein value observed at 8 and 12 hours only 79% of the total nitrogen. The nitrogen equilibrium in the cells was still strongly directed toward the synthesis of protein, and at the end of 48 hours protein nitrogen had increased to its initial 93 per cent. During the lag in protein synthesis, accumulation of soluble intermediates was observed. Increases in all fractions were found, but nitrate, amide, and ammonia nitrogen never accounted for more than about 5% of the soluble nitrogen. These substances are apparently readily utilized by the cellular machinery. Free and combined amino nitrogen also increased, and accounted for 30% of the soluble nitrogen. The most striking increase, however, was observed in the basic nitrogen fraction, which accounted for 60% of the soluble nitrogen after 12 hours. Further work showed that most of the basic nitrogen was not amino nitrogen. Thus the synthesis of the basi amino acid arginine was indicated, rather than an increase in lysine, histidine, or ornithine, Analyses for arigine by the colorimetric Sakaguchi test further substantiated this point. A reasonably close correspondence between the amount of non-amino basic nitrogen and the amount of guanido nitrogen of arginine was found. Further identification of arginine was achieved by the use of paper chromatography. Ascending one-dimensional chromatography with known compounds having a guanido group such as arginine, guanidine, and glycocyamine was carried out using phenol-water, 77% alcohol, and n-butanol-acetic acid solvent systems. The guanido compounds were detected by applying the Sakaguchi reagents to the paper. Guanidine and glycocyamine were eliminated as possible intermediates since their R_f values did not correspond to that of the unknown and the colors produced with the Sakaguchi reagents on paper differed. The color produced corresponded to that of arginine and R_f values of the unknown and arginine were the same when phenol-water and 77% ethanol were used as solvent systems. With butanol-acetic acid, however, the R_f of the unknown was somewhat less than that of arginine and was

increased when the unknown was hydrolyzed. Thus a possibility exists that an arginine peptide was formed.

Other experiments were carried out with nitrogen deficient cells in order to determine the possible precursors of arginine. If a compound were a metabolic precursor to arginine, the addition of that compound would stimulate the production of arginine. Cells were harvested from the mass culture apparatus and resuspended in nitrogen deficient nutrient solution. Aliquots of this suspension were transferred to 500 ml. flasks. To each flask 2.5 millimoles of potassium nitrate were added plus 0.125 millimoles of a possible precursor such as ornithine, urea, or citrulline. To another flask 0.125 millimoles of glutamic acid was added, since this compound might be a precursor of ornithine, and in turn, arginine. The results of these experiments are shown in Table VI.

Table VI

Effect of possible precursors on the synthesis of arginine by nitrogen deficient Scenedesmus cells.

Treatment	3 Hours	Micromoles arginine per gram dry weight 6 Hours	9 Hours	12 Hours
KNO_3 control	26	73	100	107
KCl control	-	-	-	3
KNO_3 + urea	53	96	109	118
KNO_3 + ornithine	46	90	87	114
KNO_3 + citrulline	28	77	96	111
KNO_3 + glutamate	10	24	49	90

Synthesis of arginine was definitely stimulated by two intermediates in the Krebs-Henseleit urea cycle, ornithine and urea, but stimulation was not observed when another intermediate in the cycle, citrulline, was added. Whole cells, however, are not generally permeable to citrulline. It is probable that arginine formation occurs through a reversal of this urea cycle. It is not possible at this time to explain the inhibition of arginine synthesis by glutamic acid. It was suspected that glutamate inhibited by promoting the formation of glutamine, but no increase in glutamine content due to the presence of glutamate was found when amide analyses were made on the cell extracts.

During these experiments, formation of nitrite by the cells was observed. After 12 hours approximately twenty times more nitrite was found in the medium when cells were supplied nitrate than in the potassium chloride control. Presumably, therefore nitrate is being reduced to nitrite, one of the intermediates in nitrate assimilation.

Carbohydrate deficiency

During the next experiment Scenedesmus was cultured in the dark for 13 days. Samples were taken and nitrogen fractions were determined in the usual manner. Analyses have not yet been completed, but the results obtained so far are presented in Table VII.

Table VII

Changes in the nitrogen fractions of <u>Scenedesmus</u> during a prolonged dark period producing a carbohydrate deficiency.

Fraction	Days in Darkness									
	0	1/3	2/3	1	1.5	2	3	6	10	13
	% Dry weight									
Total N	9.34	9.45	9.85	9.60	9.55	10.93	10.27	10.36	9.58	9.50
Protein N	8.87	8.95	9.43	9.04	9.01	10.21	9.62	9.41	8.68	8.51
Soluble N	0.466	0.495	0.430	0.562	0.540	0.719	0.629	0.945	0.905	0.987
Amide and NH_3N	0.015	0.017	0.013	0.021	0.022	0.023	0.025	0.041	0.087	0.087
Basic N	0.167	0.163	0.173	0.253	0.261	0.304	0.288	0.442	0.351	0.414

The increase in total nitrogen on a dry weight basis during the first three days reflects a decrease in reserve materials such as carbohydrates or fat as these materials are respired and not replaced by photosynthesis. Soluble and protein nitrogen increase proportionally also. After the first three days very slow protein breakdown occurs, decreasing from 93% of the total nitrogen to 90% at the end of 13 days. Soluble nitrogen continues to increase until the sixth day and after this time remains constant. It would appear that after 6 days in the dark a balance is set up, in which protein is broken down slowly to soluble nitrogen and these latter compounds are slowly utilized as substrates for the energy-producing system of the cell, and consequently do not build up to any appreciable extent. As in nitrogen deficiency, the shift toward proteolysis is never appreciable, thus indicating that the algal cells have a powerful system for conserving their protein when subjected to abnormal conditions.

At the present time, nitrogen fraction analyses are being completed. An additional experiment has been started to determine the effects of potassium deficiency on nitrogen metabolism. It is hoped that some insight into a function of potassium in the alga will be obtained, as well as further data on the general course of nitrogen metabolism

Experiments are also planned in which phosphorus deficiency will be studied, In view of the necessity of phosphorus to the energy-producing and utilizing systems of all cells, it is expected that a drastic shift of nitrogen from protein synthesis will occur and that information will be gained concerning the synthesis and breakdown of protein.

<u>Plans for the Future</u>

The immediate plans for future experiments are included in the discussions of the several phases of research receiving our present attention and will not be repeated here. Our long range view we hope to present in detail in a request for continuation of support to be submitted in February 1954. In brief we are looking forward to a more intensive study of the metabolism of the algae by both the direct analytical technique using mass cultures and the indirect manometric technique using metabolic inhibitors.

It is felt that a coupling of these two methods will provide tests of hypotheses formerly subject only to manometric interpretation. We believe that the quantitative phase of our investigation of algal growth in mass cultures will have reached the stage where we can rely enough on routine culture methods to provide reproducible and homogeneous samples of algal for qualitative studies of metabolism.

Reports and Publications

Publications

Krauss, Robert W. 1953. Inorganic Nutrition of Algae. p 85-102, in Algal Culture From Laboratory to Pilot Plant, ed. by John S. Burlew, Carnegie Institution of Washington, Washington, D.C.

Krauss, Robert W. and William J. McAleer. 1953. Growth and Evaluation of Species of Algae With Regard to Sterol Content. p 316-325 in Algal Culture From Laboratory to Pilot Plant, ed. by John S. Burlew, Charnegie Institution of Washington, Washington, D.C.

In Press

Krauss, Robert W. and William H. Thomas (due in March 1954) Growth and Inorganic Nutrition of _Scenedesmus obliquus_ in mass cultures. Plant Physiology.

Papers

Krauss, Robert W. 1953. Problems in Nutrient Supply for Large Scale Algal Cultures. Invitational paper presented to a joint symposium of the Botanical Society of America and the American Society of Plant Physiologists at the national meeting of the American Association for the Advancement of Science. Boston, December 29, 1953.